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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Xiang-Yang	LIU	Singapore
Yan Wei	JIA	Singapore

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

METHOD FOR PREDICTING DE NOVO BIOMACROMOLECULE CRYSTALLIZATION CONDITIONS AND FOR CRYSTALLIZATION OF THE SAME

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☒ Customer Number 27572

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☐ Firm *or* Individual Name	Harness, Dickey & Pierce, P.L.C.				
Address	P.O. Box 828				
Address					
City	Bloomfield Hills	State	MI	ZIP	48098
Country	USA	Telephone	248-641-1600	Fax	248-641-0270

ENCLOSED APPLICATION PARTS *(check all that apply)*

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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

FILING FEE AMOUNT ($) 80.00

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____.

Respectfully submitted,

SIGNATURE _____ Date 02/25/04

TYPED or PRINTED NAME Bryant E. Wade REGISTRATION NO. (*if appropriate*) 40,344

TELEPHONE (248) 641-1600 Docket Number: 2500-000032

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

EV 406 074 594 US

METHOD FOR PREDICTING DE NOVO BIOMACROMOLECULE CRYSTALLIZATION CONDITIONS AND FOR CRYSTALLIZATION OF THE SAME

Field of Invention

This invention relates to a de novo method of predicting crystallization conditions. In particular, the invention relates to a method of predicting the conditions for crystallizing biomacromolecules from solution, and more specifically for crystallizing proteins from solution.

Background

In most cases of protein crystallization experiments, the final product is not a single crystal but amorphous aggregation. To predict the likelihood of the formation of either a crystal or an amorphous aggregation, the second virial coefficient B_{22} is customarily employed by many groups [1—5]. Serving as an indicator of intermolecular interactions, B_{22} is positive when these interactions are repulsive, and negative when these interactions are attractive. As a consequence, a necessary condition for crystallization is obtained when the second virial coefficient B_{22} lies in a so-called "crystallization window": $-10^{-4} > B_{22} > -8 \times 10^{-4}$ ml mol/g^2.

The criterion based on the second virial coefficient has its advantages, as it gives a discriminating response. However this criterion does not work in some cases in which B_{22} could well lie within the crystallization window, but the experiment gives amorphous aggregation. One of the reasons for this failure is that the criterion provided by the second virial coefficient takes into account only the interactions between the biomacromolecules. However, such intermolecular interactions determine biomacromolecule crystallization only partially. Apart from this, the B_{22} criterion can only be applied at very low protein concentrations, whereas it is invalid at high protein concentrations.

The crystallization of biomacromolecules involves a nucleation and growth process, determined to a large extent by kinetics. Kinetics refers to the way biomacromolecules move in a solution, the rate at which they are transported, and the way they are incorporated in the biomacromolecule crystals at the crystal surface. The crystallization window provided by the second virial coefficient disregards kinetic and other factors, that are unrelated to intermolecular interactions but nevertheless largely influence crystallization.

Alternative methods have been developed to circumvent the drawbacks presented by the use of the second virial coefficient in attempting to predict crystallization. These methods, the so-called high throughput screening methods, are solely empirical involving a large number of solution matrices. These methods are costly, cumbersome, and time-consuming; they require large investments in expensive robots. As protein crystallization is normally a lengthy process and can be affected by about 20 different parameters, these methods have in many cases no general applicability and suffer from a low success rate.

What is needed is a de novo method with a more general applicability that is simple, easy to apply, is not wasteful on protein, does not require complex equipment, does not rely on heavy investments for its application, can be utilized by both the institutional and industrial establishments, offers substantial automation advantages, and is much less costly than the methods recited in the prior art.

Summary

A de novo method for predicting crystallization conditions for biomacromolecules is provided, giving a more reliable prediction criterion than has been possible thus far. Unlike the second virial coefficient B_{22} used to predict crystallization conditions based solely on intermolecular interactions, the present invention combines information on both the intermolecular interactions and kinetic effects to prescribe crystallization conditions. The improvement above the empirical methods available in the prior art and the prediction methods provided by calculating the second virial coefficient, is due to the incorporation of the kinetic effects that largely determine biomacromolecule crystallization, and offers the following advantages: (1) only small amounts of protein are required; (2) the measurements are much quicker than either through use of the B_{22} approach or through use of the empirical screening methods; (3) hardly any limitation needs to be applied on the protein concentration. (The B_{22} approach can only be applied to solutions with very low protein concentrations); (4) the simplicity of the required surface tension measurement of the biomacromolecule solution, facilitates the handling in an ordinary laboratory.

One object of the present invention is to identify the phase boundary between a crystal phase and a liquid phase by determining the biomacromolecule solubility, under predetermined experimental

conditions. Another object of the invention is to identify the boundary between a crystallization and an aggregation.

Therefore in accordance with a first aspect of the invention there is disclosed:

A method for predicting a crystal equilibrium condition for biomacromolecule crystallization and for crystallizing a biomacromolecule, comprising

setting up at least one crystal equilibrium condition experiment, the crystal equilibrium condition experiment comprising the steps of

a) preparing a solution comprising the biomacromolecule,

b) selecting a variable quantity,

c) selecting an assembly parameter,

d) monitoring a response of the assembly parameter while varying the variable quantity in a suitable way so that the response exhibits a transition,

e) defining a crystal equilibrium condition based on the transition,

and

crystallizing the biomacromolecule.

In accordance with a second aspect of the invention there is disclosed:

A method for predicting a boundary aggregation condition for biomacromolecule crystallization and for crystallizing a biomacromolecule, comprising

setting up at least one aggregation boundary condition experiment, the aggregation boundary condition experiment comprising

a) preparing a solution comprising the biomacromolecule,

b) selecting a variable quantity,

c) selecting an assembly parameter,

d) measuring the assembly parameter at different times,

e) registering an equilibrium assembly parameter

f) deriving a crystallization coefficient from the equilibrium assembly parameter, the crystallization coefficient being associated with the variable quantity,

g) using an aggregation indicator to define an aggregation boundary condition for the biomacromolecule, the aggregation boundary condition prescribing that an aggregation occurs

when the crystallization coefficient associated with the variable quantity is larger than the aggregation indicator,

and

crystallizing the biomacromolecule.

Brief Description of the Drawings

Embodiments of the present invention are illustrated by the following drawings:

FIGs. 1 is an illustration of predictive conditions;

FIG. 1A is an illustration of a crystal equilibrium condition;

FIG. 1B is an illustration of an aggregation boundary condition;

FIG. 2 is an illustration of a crystallization process from a solution of biomacromolecules, involving a transition from a liquid phase to an orderly solid phase, that is a crystal;

FIG. 3 is an illustration of an amorphous aggregation process involving the transition from the liquid phase to a disorderly solid phase;

FIG. 4 is an illustration of analogous processes to the crystallization and the amorphous aggregation that take place in the solution;

FIG. 5 is an illustration of a response of an assembly parameter to a tendency of the biomacromolecules to assembly;

FIG. 6 is an illustration of the way a solubility curve relates to the crystal equilibrium condition.

FIG. 7 shows plots of the assembly parameter against time;

FIG. 8 shows plots of the assembly parameter against the square root of the time;

FIG. 9 shows plots of the logarithm of the time derivative of the assembly parameter;

FIG. 10 is an illustration of a configurational rearrangement dependence on time;

FIG. 11 shows plots of a diffusion time, a penetration time and a rearrangement time; and

FIG. 12 shows a histogram illustrating how crystallization coefficients relate to the aggregation boundary condition by prescribing an aggregation indicator.

Detailed Description

The primary features of a first and a second embodiment of the invention of a method of predicting biomacromolecule crystallization conditions and for crystallizing biomacromolecules are provided hereinafter with reference to FIG. 1A and FIGs. 2--6, whereas a detailed account thereof is provided hereinafter with reference to FIG. 1B, FIGs. 2–5 and FIGs. 7-12. In the first embodiment, a crystal equilibrium condition in FIG. 1A is expressed by means of a curve serving as a boundary separating two regions of experimental parameter values: a region where crystallization can occur, and a region where crystallization cannot occur. In a second embodiment, an aggregation boundary condition in FIG. 1B is expressed by means of a window of experimental parameter values above which an amorphous aggregation is likely to occur.

Theoretical Introduction

In attempts to crystallize biomacromolecules from a solution, it is desirable to obtain as much as possible single crystals with as few defects as possible, and to avoid amorphous aggregations of molecules, since amorphous aggregates are not crystals. The present invention takes advantage of the property of biomacromolecules to have mixed hydrophobic and hydrophilic regions. This property results in a tendency for these molecules to assembly either in the bulk or at the surface of the solution. In this disclosure, the surface of the solution can be adjacent to another material or to empty space, and hence the surface can be in contact with a solid or with a liquid or with a gas, that is usually air. The surface of the solution has a surface tension and a surface pressure, which terms in this case include an interfacial tension or an interfacial pressure.

It is possible to define one or more assembly parameters that reach a critical response as increasingly more molecules participate in assembly formation. For example, the tendency of biomacromolecules to assembly in a solution can be monitored by taking density, conductivity, detergency, osmotic pressure, surface tension or surface pressure measurements of the solution.

Biomacromolecule crystallization conditions are reflected in the tendency of biomacromolecules to assembly. The crystallization conditions and the assembly characteristics of biomacromolecules are governed by both the intermolecular forces and the kinetic effects, depending on the experimental situation. The present invention prescribes two procedures of measuring the assembly

parameters in solution so as to determine reliable crystallization conditions without the prior need to carry out crystallization experiments.

Application

In FIG. 2 a crystallization process from the solution 120 comprising biomacromolecules 130 involves a transition 122 from a liquid phase 124 to an orderly solid phase, that is a crystal 126, wherein solid-phase biomacromolecules 132 are regularly arrayed and regularly oriented. The biomacromolecules 130 in the liquid phase 124, become adsorbed biomacromolecules 136 on a surface 138 of the crystal 126, and subsequently they acquire a suitable orientation 140 in order to become incorporated as in the crystal 126 as the solid-phase biomacromolecules 132. The crystallization occurs because a transport time, being an amount of time required for the biomacromolecules 130 in the solution 120 to reach the surface 138 of the crystal 126, otherwise known as a diffusion time t_{diff}, is longer than an integration time t_{int}, wherein the integration time is an amount of time required for the adsorbed biomacromolecules 136 to become the solid-phase biomacromolecules 132, and it includes an amount of time required for their rearrangement.

In FIG. 3 on the other hand, the amorphous aggregation involves the transition 122 from the liquid phase 124 to a disorderly solid phase 128, wherein aggregate biomacromolecules 134 have irregular positions and irregular orientations. The amorphous aggregation occurs because the diffusion time t_{diff} required for the biomacromolecules 130 in the solution 120 to reach the solid phase 128 is shorter than the time required for their rearrangement and integration.

In FIGs. 2 and 3 the solution 120 has an appropriate temperature and an appropriate pH. In the invention there is no restriction on the appropriate temperature, which is usually taken to be around a room temperature. A buffering material may be introduced in the solution 120 to maintain a desirable level of the pH. The solution 120 can also comprise additives like small or large organic molecules or salt. By way of example, the biomacromolecule 130 in FIGs. 2 and 3 is a protein, more specifically lysozyme, and the additive is a salt, more specifically NaCl.

FIG. 4 illustrates that processes analogous to the crystallization or the amorphous aggregation take place in the solution 120 containing the biomacromolecules 130. The solution 120 is placed in a container 140, and the solution has a surface 152, which in this application is adjacent to air.

During a diffusion step 142, the biomacromolecules 130 take an average amount of time, that is the diffusion time t_{diff}, to diffuse 148 towards the surface 152. Subsequently, during a penetration step 144, the biomacromolecules 130 take an average amount of time, that is a penetration time t_{pen}, to penetrate 150 the surface 152. Subsequently, during a rearrangement step 146, the biomacromolecules 130, that penetrated 150 the surface 152 take an average amount of time, that is a rearrangement time t_{arr}, to undergo the rearrangement 154 and finally become integrated in the surface 152. Thus the integration time t_{int} is the sum of the penetration time and the rearrangement time is $t_{int} = t_{pen} + t_{arr}$, because it equals an amount of time required to complete the penetration step 144 and the rearrangement step 146.

In FIG. 5 the biomacromolecules 130 have a biomacromolecule concentration 156, and the additives have an additive concentration 157. Without limitation to the scope of the invention, the biomacromolecule concentration 156 is taken, e.g. 4 mg/ml lysozyme, in some experimental runs, while the additive (salt NaCl) concentration 157 is allowed to vary. In some other experimental runs the additive concentration 157 is taken, e.g. 1 M NaCl, while the biomacromolecule concentration 156 is allowed to vary.

Referring to FIGs. 5 an 6, in the first embodiment of the invention, measurements of an assembly parameter 164 (for example a surface tension or a surface pressure) with respect to a variable quantity 158 (for example the biomacromolecule concentration 156, the additive concentration 157 or the pH), are utilized to express the crystal equilibrium condition. According to that condition, as will be explained further on, in FIG. 1A (FIG. 6), the crystallization is expected in a region 172 delineated by a solubility curve 170.

A probability that the aggregation (FIG. 3) will dominate above the crystallization (FIG. 2), increases when the integration time t_{int} is substantially longer than the diffusion time t_{diff}. That probability can be quantified as illustrated in FIG. 1B (FIG. 12), by means of a crystallization coefficient 232 $k_{cryst} = t_{int}/t_{diff}$ defined as a ratio of the integration time to the diffusion time. An aggregation indicator resulting from the crystallization coefficient should therefore lie certainly above $k_{cryst} = 1$.

Because the crystallization coefficient 232 is a dimensionless ratio, it is expected to have general applicability for most biomacromolecules. The method is illustrated in this experiment by

choosing as an example the protein lysozyme to serve as a model system for the biomacromolecule crystallization. It is therefore expected that the aggregation indicator obtained from the crystallization coefficient derived for lysozyme will serve as a standard criterion to define the aggregation boundary condition for the crystallization of most biomacromolecules.

In the second embodiment of the invention, measurements of the diffusion time t_{diff}, the penetration time t_{pen} and the rearrangement time t_{arr} are used to calculate the crystallization coefficient 232 $k_{cryst} = t_{int}/t_{diff}$ which in turn is used to express the aggregation boundary condition, as explained further below with reference to FIGs. 7–12.

Crystal Equilibrium Condition

In the first embodiment of the invention, in FIGs. 4—6, the crystal equilibrium condition is determined by constructing the solubility curve 170.

FIG. 5 illustrates a response of the assembly parameter 164 to the activity of the biomacromolecules 130 in the solution 120. The suitable assembly parameter 164 for this application is the surface tension or the surface pressure, and in this case it is taken to be the surface tension, that can be measured with a tensiometer. A suitable variable quantity 158 for this application is the biomacromolecule concentration 156 or the additive concentration 157 or the pH. When a suitable variable quantity 158 has benn selected, it can be made to vary in order to sample the response of the assembly parameter 164. In this case the variable quantity 158 is taken as the biomacromolecule concentration 156, whereas the additive concentration 157 is taken to be 1M NaCl.

FIG. 5 shows a plot of the assembly parameter 164 (the surface tension) against the logarithm 159 of the variable quantity 158 (the biomacromolecule concentration 156), according to which the assembly parameter 164 decreases as the variable quantity 158 is gradually increased. This is illustrated by the diffusion step 142, followed by the penetration step 144, subsequently resulting in completion of the rearrangement step 146. Upon completion of the rearrangement step 146, a complete layer of rearranged biomacromolecules has been assembled at the surface 152, allowing no space for any more biomacromolecules 130 to penetrate the surface 152. Therefore a further increase of the variable quantity 158, being in this experiment the biomacromolecule concentration,

cannot cause a further decrease in the assembly parameter 164, being in this experiment the surface tension, but will instead result in accumulation of biomacromolecules 130 in the bulk of the solution 120.

Thus in FIG. 5 as more and more biomacromolecules 130 tend towards assembly formation, the response of the assembly parameter 164 shows a transition 162 between a changing response 161 and a substantially constant response 163 of the assembly parameter 164. The transition 162 is associated not only with a critical response of the assembly parameter 164, but also with a critical magnitude 165 of the variable quantity 158. In this case the critical response of the assembly parameter164 is equal to the surface tension of 53 mN/m. The critical magnitude 165 of the variable quantity 158 is equal to the lysozyme concentration of 4mg/ml; this corresponds to the logarithm 159 of the variable quantity 158 equal to 1.4, when the additive concentration 157 is equal to 1 M NaCl.

Because in this particular example the assembly parameter 164 is the surface tension, it decreases as the variable quantity 158, that is the biomacromolecule concentration 156, is increased, and hence the critical response of the assembly parameter 164 is substantially minimal. The crystal equilibrium condition can be expressed by means of the critical magnitude 165 of the variable quantity 158: when the temperature and pH are held at their predetermined values, and when the salt concentration is held at 1M NaCl, then the crystallization cannot occur for values of the lysozyme concentration 156 falling below 4 mg/ml. A similar critical behavior is observed when the additive concentration 157 is allowed to vary, while the biomacromolecule concentration 156 is held constant at 14 mg/ml lysozyme (drawing not shown).

The aforementioned behavior should not be construed to be typical of a general case covered by the scope of the invention in which the transition 162 refers to the changing response and the substantially stable or the substantially unchanging response, considering that the changing response need not imply a purely decreasing or a purely increasing response.

FIG. 6 illustrates how the solubility curve 170 can be constructed in order to express the crystal equilibrium condition. The solubility curve 170 follows when two of the following quantities: the biomacromolecule concentration 156, the additive concentration 157 and the pH, are allowed to form a pair of the variable quantities. In the example of FIG. 6 the biomacromolecule

concentration 156 and the additive concentration 157 form the pair of the variable quantities, while the pH is kept fixed. A series of transitions 162 is obtained, each transition associated with the critical response of the assembly parameter 164. In the particular experiment described here, four transitions 162 were measured and employed in order to construct the solubility curve 170. For example, a point 171 on the solubility curve 170 indicates the following pair of the variable quantities: the biomacromolecule concentration 156 is 4 mg/ml lysozyme while the additive concentration 157 is around 0.65 M NaCl. The corresponding crystal equilibrium condition follows by specifying that the crystallization can occur in a crystallization region 172 in which either one of the pair of the variable quantities assumes values at or above the corresponding critical magnitude 165 on the solubility curve 170. Therefore the crystal equilibrium condition as illustrated in FIG. 6 is obtained by recording a series of the critical magnitudes 165 of the variable quantity and the corresponding critical response of the assembly parameter 164, as illustrated in FIG. 5.

For the measurement of the points on the solubility curve the Wilhelmy plate method is employed using a K14 Kruss tensiometer, according to the following steps.

1. A buffer solution is prepared at the predetermined pH. A biomacromolecule stock solution is prepared by dissolving the biomacromolecule in the buffer solution. An additive stock solution is prepared by dissolving the additive in the buffer solution.

2. In FIG. 5, various solutions 120 are prepared at the predetermined temperature, usually room temperature, having the additive concentration 157 and different biomacromolecule concentrations 156 starting from 0 and increasing as long as the solutions 120 maintain a clear appearance. The solutions 120 are kept at the predetermined temperature for a few hours. The surface tension or surface pressure 164 of each solution 120 is measured by putting it into the Kruss tensiometer until the surface tension or pressure reaches a constant value. The chamber of the tensiometer is saturated with pure water vapor to maintain a uniform humidity, and the solutions 120 are kept at the predetermined temperature.

The surface tension or surface pressure 164 is recorded and plotted against the biomacromolecule concentration 156. The critical magnitude 165 of the biomacromolecule concentration 156 occurring at the critical point 162 is registered. It corresponds to the surface tension or surface pressure 164, undergoing the transition 162 from the changing response 161 to

the substantially constant response 163 as the biomacromolecule concentration 156 increases. The critical point 162 determined in this way is a solubility value of the biomacromolecule at the additive concentration 157.

Step 2 is repeated to obtain solubility points at different additive concentrations 157. Steps 1 and 2 are repeated to find the solubility values at different additive concentration 157 and different pH.

Aggregation Boundary Condition

In the second embodiment of the invention, the measurement of the aggregation boundary condition is carried out, as illustrated in FIGs. 4 and 5, by the following steps.

1. The buffer solution is prepared at the predetermined pH. The biomacromolecule stock solution is prepared by dissolving the biomacromolecule in the buffer solution. The additive stock solution is prepared by dissolving the additive in the buffer solution.

2. Various solutions 120 are prepared having the biomacromolecule concentration 156 and different additive concentrations 157. In the particular experiment described here the biomacromolecule concentration is 1 mg/ml lysozyme. Immediately after mixing each of the solutions 120, the surface tension is measured with respect to time and recorded. The measurement is stopped when the surface tension in each solution reaches a constant value. The obtained data are analyzed according to the steps outlined in FIGS. 7--12 described below.

Step 2 is repeated for different pH.

In the particular application, and without limitation to the scope of the invention, the experiment was carried out at the room temperature of 23°C. In FIGs. 4 and 5 the biomacromolecule 130 concentration is taken 1 mg/ml of lysozyme, the pH quantity is 4.5 and the buffering agent is 50 mM sodium acetate. In FIGs. 7—10 the additive is the salt NaCl, and the variable quantity 158, taken to be the additive concentration 157, is varied from 0 to 2.4 M in steps of 0.4 M. In FIGs. 7 and 8 the assembly parameter 164 is taken to be the surface tension s. In FIGs. 9 and 10 the assembly parameter 164 is the surface pressure p related to the surface tension s by the equation: $p = s - s_0$, where s_0 is the surface tension of the solvent in the absence of biomacromolecules. For clarity, and without limitation to the scope of the invention, in FIGs. 7—10

only three values of the additive concentration 157 are used to illustrate the second embodiment of the invention: 0.0 M NaCl 182, 0.4 M NaCl 184 and 1.6 M NaCl 186.

FIG. 7 shows plots 191, 192, 193 of the assembly parameter 164 taken to be the surface tension s, against time 180, for, respectively, the three additive concentrations 182, 184, 186. We see that the corresponding assembly parameters 164 remain unchanged when equilibrium times t_{eq} 194, 195 and 196 are reached. Each equilibrium time t_{eq} corresponds to an equilibrium surface tension s_{eq}.

FIG. 8 shows plots 197, 198, 199 of the assembly parameter 164 against the square root of the time 180 for, respectively, the three additive concentrations 182, 184, 186. FIG. 9 shows plots 202, 204, 206 of the logarithm of the time derivative of the assembly parameter 164, taken to be the surface pressure p, against the surface pressure p, for , respectively, the three additive concentrations 182, 184, 186. FIG. 10 illustrates a configurational rearrangement time dependence 220 against time 180, which expression in the present application is given by $\ln(1-p/p_{eq})$, where ln is the natural logarithm, p is the surface pressure and p_{eq} is an equilibrium surface pressure.

In plots 212, 214, 216 of the configurational rearrangement expression 220 the equilibrium surface pressures p_{eq} are equal to the surface pressure evaluated, respectively, at the equilibrium times t_{eq} 194, 195, 196 in FIG. 7. The equilibrium surface pressure is given by $p_{eq} = s_{eq} - s_0$, where s_{eq} is the equilibrium surface tension.

We notice that each plot in FIGs. 8, 9 and 10 exhibits three line segments identifying the steps 142 144 and 146 in FIGs. 4 and 5: a first straight line segment 221 identifies the diffusion step 142, a second straight line segment 222 identifies the penetration step 144, and a third straight line segment 223 identifies the rearrangement step 146. In FIG. 10 the diffusion time t_{diff} is calculated by the inverse of the slope of the first straight line segment 221, the penetration time t_{pen} is calculated by the inverse of the slope of the second straight line segment 222, and the rearrangement time t_{arr} is calculated by the inverse of the slope of the third straight line segment 223, so that the integration time is obtained as a sum of the last two, $t_{int} = t_{pen} + t_{arr}$.

FIG. 11 shows a plot 224 of the diffusion time t_{diff}, a plot 226 of the penetration time t_{pen}, a plot 228 of the rearrangement time t_{arr}, and a plot 230 of the integration time $t_{int} = t_{pen} + t_{arr}$, against the additive concentration 157. In this experiment the additive concentration 157 is taken in a range

from 0 to 2.4 M NaCl in steps of 0.4 M NaCl. A histogram in FIG. 12 illustrates how the crystallization coefficients 232 relate to the aggregation boundary condition. The crystallization coefficients 232 $k_{cryst} = t_{int}/t_{diff}$, are calculated for all pairs of obtained values for the integration time t_{int} and the diffusion time t_{diff}, and are shown against the additive concentration 157 in the above range.

Subsequently crystallization experiments were carried in order to quantify the aggregation boundary condition in terms of the crystallization coefficient k_{cryst} 232. The aforementioned range of the additive concentration 157 was used, while varying the biomacromolecule concentration 156 in a range 5--100 mg/ml lysozyme. The crystallization results are classified in three domains 234 listed below and included in FIG. 12, where the crystallization coefficient 232 is shown against the additive concentration 157.

Domain A: neither crystallization nor aggregation occurs in the entire range of the biomacromolecule concentration (lys, in mg/ml), at the indicated additive concentration (salt, in M NaCl);

Domain B: crystallization occurs at the indicated biomacromolecule concentration (lys, in mg/ml), at the indicated additive concentration (salt, in M NaCl) ; and

Domain C: only aggregation, but no crystallization, occurs at the indicated biomacromolecule concentration (lys, in mg/ml), at the indicated additive concentration (salt, in M NaCl).

With reference to FIG. 12, we therefore have the following table:

Domain	salt (M NaCl)	lys (mg/ml)	k_{cryst}
A	0.0	no crystallization	approx. 1.9
B	0.5	larger than approx. 70	approx. 5
B	1.0	larger than approx. 20	approx. 4.8
B	1.5	larger than approx. 10	approx. 7.8
C	2.0	only aggregation	approx. 8.5
C	2.5	only aggregation	above 11.5

The third column of this table shows the range of the lysozyme concentration used for the crystallization experiment. We see from FIG. 12 that the aggregation boundary condition prescribes that when the crystallization coefficient is larger than approximately 8, the biomacromolecule has a high probability to undergo the amorphous aggregation. Hence the crystallization is not expected for the salt concentrations around 2.0 M or above, regardless of the lysozyme concentration used. On the other hand, when the salt concentration is lower than around 0.3 M NaCl, neither the crystallization nor the aggregation is expected to occur, regardless of the lysozyme concentration used. Therefore the domain B is a window imposed on the crystallization conditions prescribed by the crystal equilibrium condition depicted in FIG. 6. Since lysozyme is a model protein system indicative of the crystallization behaviour of most biomacromolecules, the aggregation indicator is expected to be approximately 8 for most biomacromolecules.

Examples

A combination of the crystal equilibrium condition in FIG. 1A or FIG. 6 and the aggregation boundary condition in FIG. 1B or FIG. 12 is used to illustrate how crystallization and aggregation conditions can be predicted.

As a first example, the point 171 on the solubility curve 170 of FIG. 1A or FIG. 6 corresponds to a pair of the salt concentration and the lysozyme concentration: salt = 1 M NaCl, lys = 4 mg/ml. The crystal equilibrium condition is prescribing that crystallization can occur when the salt and lysozyme concentrations are taken above this pair of values, and the aggregation boundary condition confirms this.

As a second example, the aggregation boundary condition in FIG. 1B or FIG. 12 shows that when the salt concentration is taken above around 1.9 M NaCl, no crystallization may be expected to occur, regardless of the lysozyme concentration; in other words any lysozyme concentration used has a very high probability of resulting in the amorphous aggregation. The crystallization coefficient 232 associated with the pair: salt = 1 M NaCl, lys = 1 mg/ml according to the above table and in FIG. 1B or FIG. 12 is approximately 4.8. Therefore, when the salt concentration is 1 M NaCl, the crystallization should take place for some range of lysozyme concentration. Indeed, the crystallization does occur for lysozyme concentration above 20 mg/ml.

CLAIMS

1. A method for predicting a crystal equilibrium condition for biomacromolecule crystallization and for crystallizing a biomacromolecule, comprising

setting up at least one crystal equilibrium condition experiment, the crystal equilibrium condition experiment comprising the steps of

a) preparing a solution comprising the biomacromolecule,

b) selecting a variable quantity,

c) selecting an assembly parameter,

d) monitoring a response of the assembly parameter while varying the variable quantity in a suitable way so that the response exhibits a transition,

e) defining a crystal equilibrium condition based on the transition,

and

crystallizing the biomacromolecule.

2. The method as claimed in Claim 1, wherein the solution further comprises a solvent and the solution has a biomacromolecule concentration and a pH.

3. The method as claimed in Claim 2, wherein the solution further comprises an additive and the solution has an additive concentration.

4. The method as claimed in Claim 1, wherein the solution has a surface,

5. The method as claimed in Claim 2, wherein the variable quantity is one of the biomacromolecule concentration and the pH.

6. The method as claimed in Claim 3, wherein the variable quantity is one of the biomacromolecule concentration, the additive concentration and the pH.

7. The method as claimed in Claim 1, wherein the assembly parameter is one of a density, a conductivity, a detergency and an osmotic pressure.

8. The method as claimed in Claim 4, wherein the assembly parameter is one of a surface tension and a surface pressure.

9. The method as claimed in Claim 1, wherein the transition is associated with a critical magnitude of the variable quantity.

10. The method as claimed in Claim 1, wherein the transition is between a changing response of the assembly parameter and a substantially unchanging response of the assembly parameter.

11. The method as claimed in Claim 10, wherein the transition is further associated with a critical magnitude of the variable quantity.

12. The method as claimed in Claim 11, wherein the substantially unchanging response corresponds to a substantially minimal value of the assembly parameter.

13. The method as claimed in Claim 11, further defining the crystal equilibrium condition in terms of the critical magnitude.

14. The method as claimed in Claim 12, further defining the crystal equilibrium condition in terms of the critical magnitude, wherein the crystal equilibrium condition prescribes that no crystallization occurs when the variable quantity is smaller than the critical magnitude.

15. The method as claimed in Claim 1, wherein the biomacromolecule to be crystallized is a protein.

16. The method as claimed in Claim 15, wherein the protein has a weight less than 300 Dalton.

17. The method as claimed in Claim 15, wherein the protein is lysozyme.

18. The method as claimed in Claim 1, wherein the biomacromolecule to be crystallized is a polypeptide.

19. The method as claimed in Claim 1, wherein the biomacromolecule to be crystallized is a nucleic acid.

20. The method as claimed in Claim 1, wherein the biomacromolecule to be crystallized is a virus.

21. The method as claimed in Claim 1, wherein the biomacromolecule to be crystallized is a virus fragment.

22. The method as claimed in Claim 3, wherein the additive is a salt.

23. The method as claimed in Claim 3, wherein the additive is a small organic molecule.

24. The method as claimed in Claim 3, wherein the additive is a large organic molecule.

25. The biomacromolecule crystallized according to any of the Claims 1—24.

26. A method for predicting a crystal equilibrium condition for lysozyme protein crystallization and for crystallizing a lysozyme protein, comprising

setting up at least one crystal equilibrium condition experiment, the crystal equilibrium condition experiment comprising the steps of

a) preparing a solution of the lysozyme protein in a solvent, the solution further comprising a salt, the solution having a pH, a lysozyme protein concentration and a salt concentration, the solution having a surface, the surface having a surface tension and a surface pressure,

b) defining an assembly parameter to be one of the surface tension and the surface pressure,

c) selecting at least one first variable quantity and at least one second variable quantity from the lysozyme protein concentration, the salt concentration and the pH.

d) varying the at least one first variable quantity in a suitable way so that the assembly parameter exhibits a transition between a changing response and a substantially unchanging response, wherein the substantially unchanging response corresponds to a first substantially minimal value of the assembly parameter, the transition being associated with an at least one first critical magnitude of the at least one first variable quantity,

e) varying the at least one second variable quantity in a suitable way so that the assembly parameter exhibits a transition between a changing response and a substantially unchanging response, wherein the substantially unchanging response corresponds to a second substantially minimal value of the assembly parameter, the transition being associated with an at least one second critical magnitude of the at least one second variable quantity,

f) constructing a solubility curve from pairs of the at least one first critical magnitude and the at least one second critical magnitude, in order to assist in defining a crystal equilibrium condition,

g) defining the crystal equilibrium condition which is based on the solubility curve, and which prescribes that crystallization occurs when the at least one first variable quantity is not smaller than the at least one first critical magnitude, and the at least one second variable quantity is not smaller than the at least one second critical magnitude,

and

crystallizing the lysozyme protein.

27. The lysozyme protein crystallized according to Claim 26.

28. A method for predicting a boundary aggregation condition for biomacromolecule crystallization and for crystallizing a biomacromolecule, comprising

setting up at least one aggregation boundary condition experiment, the aggregation boundary condition experiment comprising

a) preparing a solution comprising the biomacromolecule,

b) selecting a variable quantity,

c) selecting an assembly parameter,

d) measuring the assembly parameter at different times,

e) registering an equilibrium assembly parameter

f) deriving a crystallization coefficient from the equilibrium assembly parameter, the crystallization coefficient being associated with the variable quantity,

g) using an aggregation indicator to define an aggregation boundary condition for the biomacromolecule, the aggregation boundary condition prescribing that an aggregation occurs when the crystallization coefficient associated with the variable quantity is larger than the aggregation indicator,

and

crystallizing the biomacromolecule.

29. The biomacromolecule crystallized according to Claim 28.

30. A method for predicting a boundary aggregation condition for biomacromolecule crystallization and for crystallizing a biomacromolecule, comprising

setting up at least one aggregation boundary condition experiment, the aggregation boundary condition experiment comprising

a) preparing a solution comprising the biomacromolecule, the solution having a surface, the surface having a surface pressure,

b) selecting a variable quantity,

c) obtaining the surface pressure at different times, while varying the variable quantity,

d) recording a time dependent equilibrium surface pressure which is associated with the variable quantity,

e) formulating a configurational rearrangement time dependence based on the equilibrium surface pressure, which is associated with the variable quantity,

f) deriving from the configurational rearrangement time dependence a crystallization coefficient of the biomacromolecule, that is associated with the variable quantity,

g) using an aggregation indicator to define an aggregation boundary condition for the biomacromolecule, the aggregation boundary condition prescribing that an aggregation occurs when the crystallization coefficient associated with the variable quantity is larger than the aggregation indicator,

and

crystallizing the biomacromolecule.

31. The method as claimed in Claim 30, wherein the solution further comprises a solvent and the solution has a biomacromolecule concentration and a pH.

32. The method as claimed in Claim 31, wherein the solution further comprises an additive and the solution has an additive concentration.

33. The method as claimed in Claim 31, wherein the variable quantity is one of the biomacromolecule concentration and the pH.

34. The method as claimed in Claim 32, wherein the variable quantity is one of the biomacromolecule concentration, the additive concentration and the pH.

35. The method as claimed in Claim 30, wherein the step of deriving the crystallization coefficient comprises the steps of

obtaining a diffusion time of the biomacromolecule,

obtaining an integration time of the biomacromolecule,

dividing the integation time by the diffusion time to obtain the crystallization coefficient of the biomacromolecule, that is associated with the variable quantity.

36. The method as claimed in Claim 30 wherein the configurational rearrangement time dependence is given by $\ln(1-p/p_{eq})$, where ln is the natural logarithm, p is the surface pressure and p_{eq} is an equilibrium surface pressure.

37. The method as claimed in Claim 36, where the step of deriving the crystallization coefficient comprises the steps of

constructing a plot of the configurational rearrangement time dependence against the time,

identifying on the plot a first substantially straight linear segment, a second substantially straight linear segment and a third substantially straight linear segment, where the second substantially straight linear segment is later in time than the first substantially straight linear segment and the second substantially straight linear segment is later in time than the third substantially straight linear segment,

equating a diffusion time to an inverse slope of the first substantially straight linear segment,

equating a penetration time to an inverse slope of the second substantially straight linear segment,

equating a rearrangement time to an inverse slope of the third substantially straight linear segment,

adding the penetration time and the rearrangement time to obtain an integration time

dividing the integration time by the diffusion time to obtain the crystallization coefficient of the biomacromolecule, that is associated with the variable quantity.

38. The method as claimed in Claim 30, wherein the biomacromolecule to be crystallized is a protein.

39. The method as claimed in Claim 38, wherein the protein has a weight less than 300 Dalton.

40. The method as claimed in Claim 39, wherein the protein is lysozyme.

41. The method as claimed in Claim 30, wherein the biomacromolecule to be crystallized is a polypeptide.

42. The method as claimed in Claim 30, wherein the biomacromolecule to be crystallized is a nucleic acid.

43. The method as claimed in Claim 30, wherein the biomacromolecule to be crystallized is a virus.

44. The method as claimed in Claim 30, wherein the biomacromolecule to be crystallized is a virus fragment.

45. The method as claimed in Claim 32, wherein the additive is a salt.

46. The method as claimed in Claim 32, wherein the additive is a small organic molecule.

47. The method as claimed in Claim 32, wherein the additive is a large organic molecule.

48. The biomacromolecule crystallized according to any one of the Claims 30—47.

49. A method for predicting a boundary aggregation condition for lysozyme protein crystallization and for crystallizing a lysozyme protein, comprising

setting up at least one aggregation boundary condition experiment, the aggregation boundary condition experiment comprising

a) preparing a solution comprising the lysozyme protein, a solvent, a salt, and a suitable buffer, the solution having a salt, a lysozyme concentration and a pH, the solution having a surface, the surface having a surface pressure,

b) obtaining the surface pressure at different times, while varying the salt concentration,

c) recording a time dependent equilibrium surface pressure, which corresponds with an equilibrium time, and which is associated with the salt concentration,

d) formulating a configurational rearrangement time dependence, which is given by $\ln(1-p/p_{eq})$, where ln is the natural logarithm, p is the surface pressure and p_{eq} is the equilibrium surface pressure, and which is associated with the salt concentration,

e) constructing a plot of the configurational rearrangement time dependence against the time,

f) identifying on the plot a first substantially straight linear segment, a second substantially straight linear segment and a third substantially straight linear segment, where the second substantially straight linear segment is later in time than the first substantially straight linear segment and the second substantially straight linear segment is later in time than the third substantially straight linear segment,

g) equating a diffusion time to an inverse slope of the first substantially straight linear segment,

h) equating a penetration time to an inverse slope of the second substantially straight linear segment,

i) equating a rearrangement time to an inverse slope of the third substantially straight linear segment,

j) adding the penetration time and the rearrangement time to obtain an integration time

k) dividing the integration time by the diffusion time to obtain the crystallization coefficient of the lysozyme protein, that is associated with the salt concentration,

g) using an aggregation indicator to define an aggregation boundary condition for the lysozyme protein, the aggregation boundary condition prescribing that an aggregation occurs when the crystallization coefficient associated with the salt concentration is larger than the aggregation indicator, wherein the aggregation indicator is within a range 7--9.

50. The lysozyme protein crystallized according to Claim 49.

Abstract

A method de novo is provided for predicting crystallization conditions and for crystallizing biomacromolecules, in particular proteins. The method provides a simple, quick and precise approach in determining the biomacromolecule solubility in different solutions, as well as the boundary between crystallization and aggregation. Because the method relies only on monitoring the assembly behavior of the biomacromolecule at the surface of a solution, it has general applicability and requires a relatively short amount of time to provide results that are reliable. Because there is no need to first crystallize the biomacromolecule, small amounts of protein are sufficient. Because the method works by measuring the surface tension or pressure of the surface of the biomacromolecule solution, it is easy, precise and quick. Furthermore it is cost-effective in requiring simple and inexpensive equipment.

Title: METHOD FOR PREDICTING DE NOVO BIOMACROMOLECULE CRYSTALLIZATION CONDITIONS AND FOR CRYSTALLIZATION OF THE SAME
Inventors: Xiang-Yang LIU, et al.
Atty. Ref.: 2500-000032

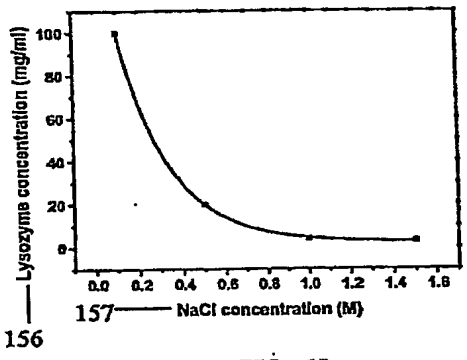

FIG. 1A

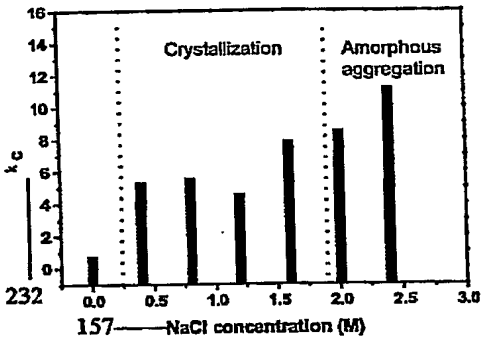

FIG. 1B

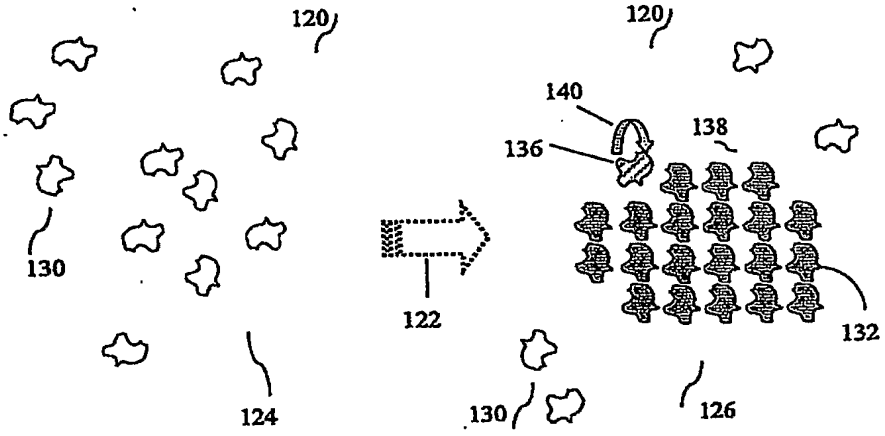

FIG. 2

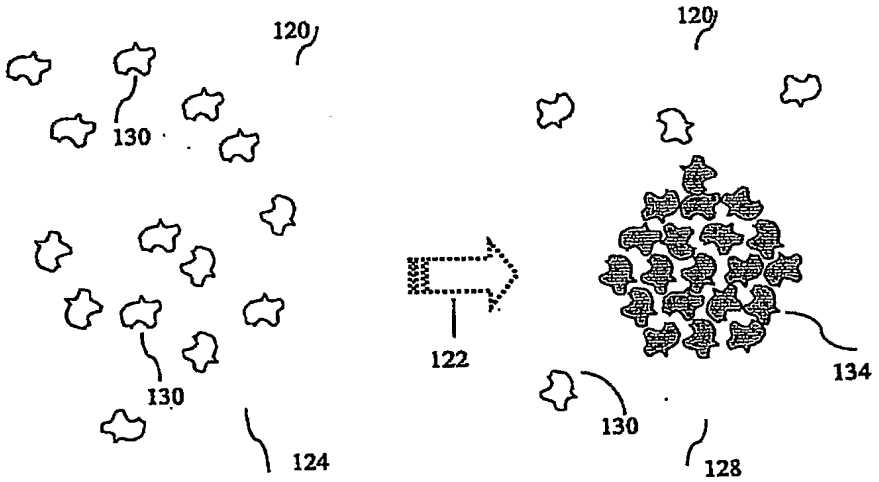

FIG. 3

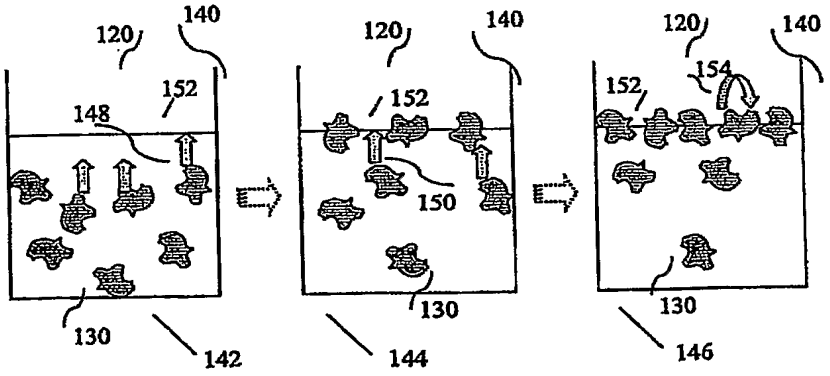

FIG. 4

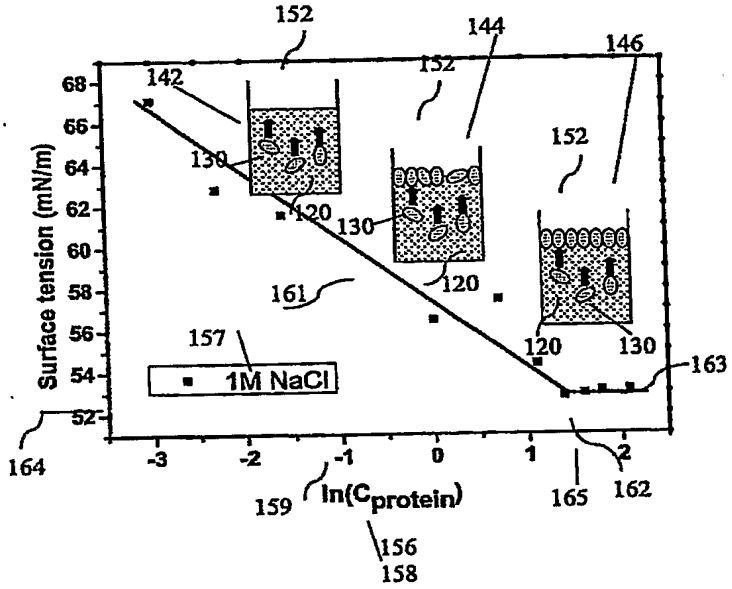

FIG. 5

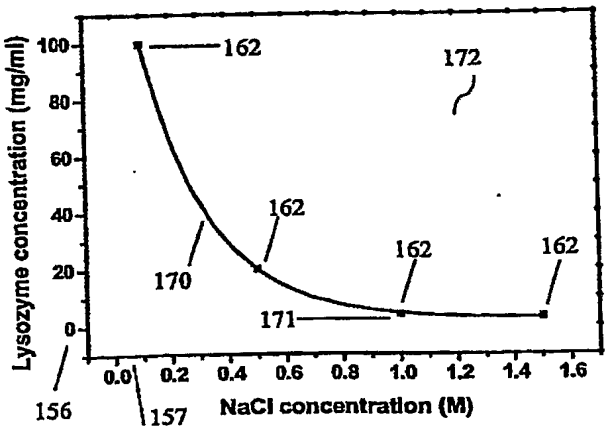

FIG. 6

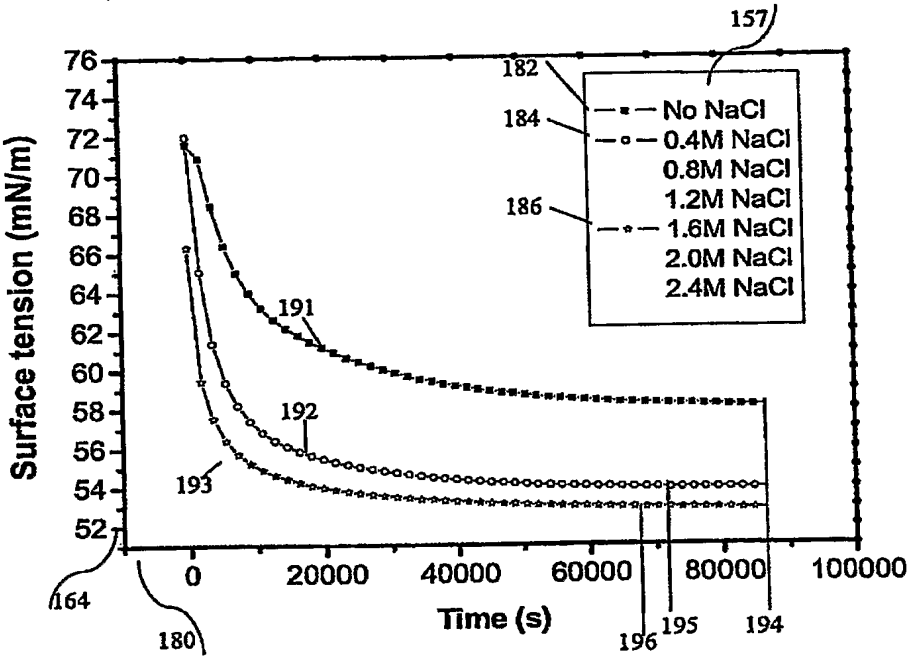

FIG. 7

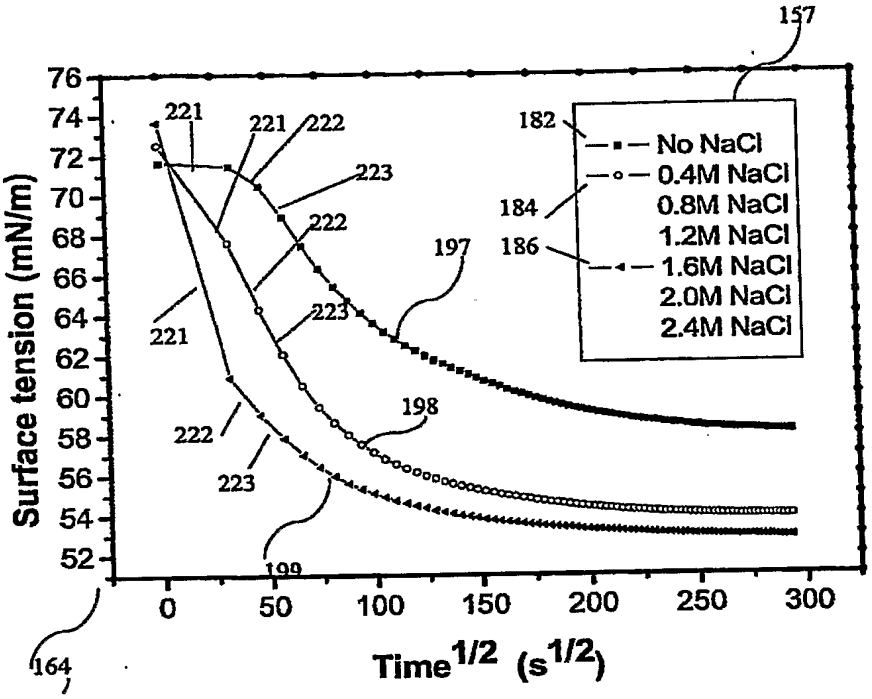

FIG. 8

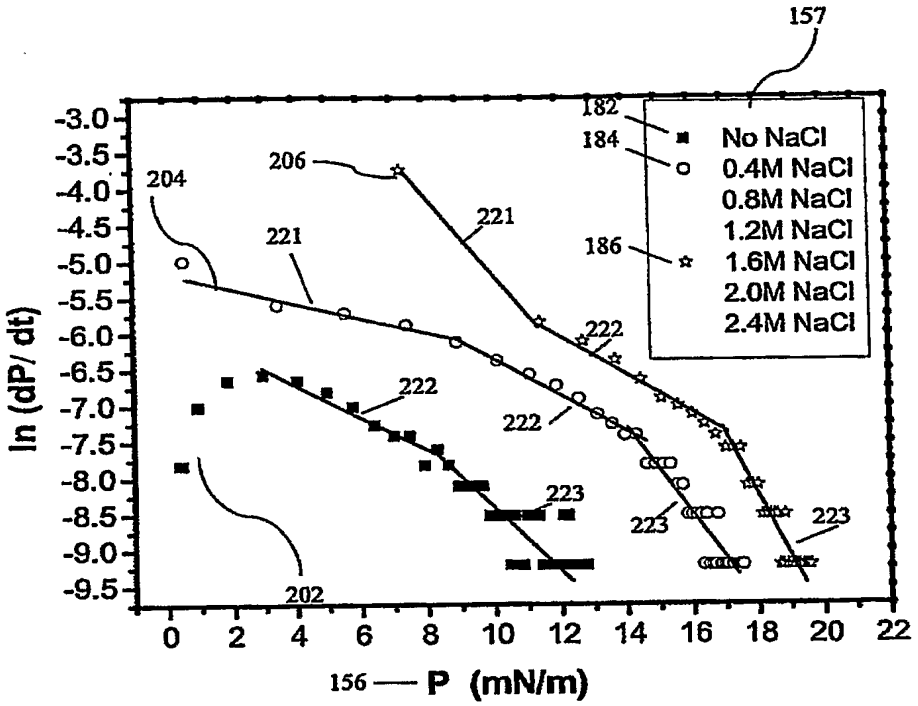

FIG. 9

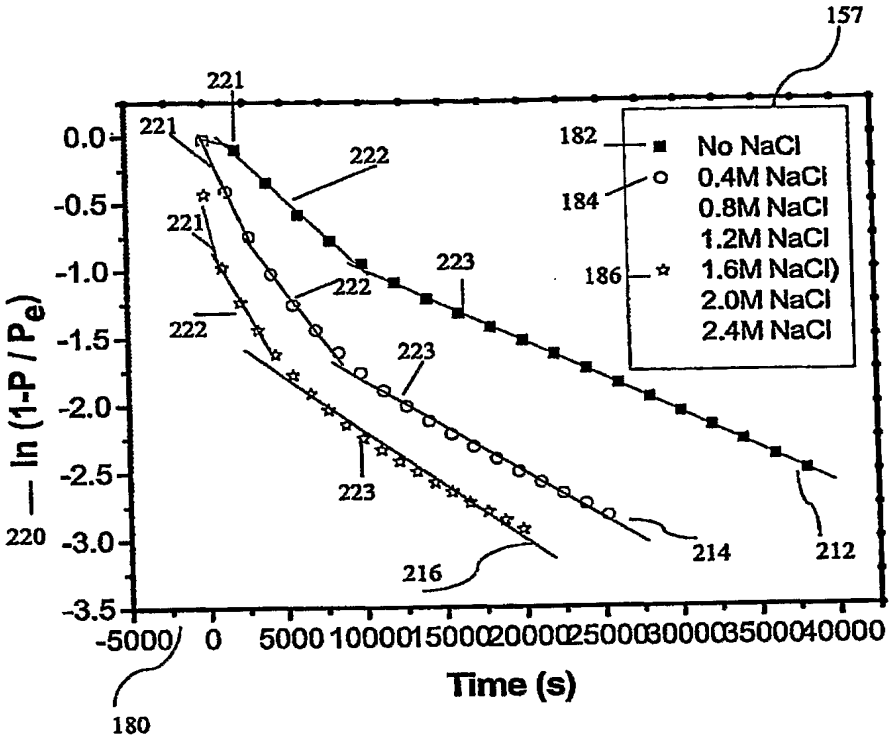

FIG. 10

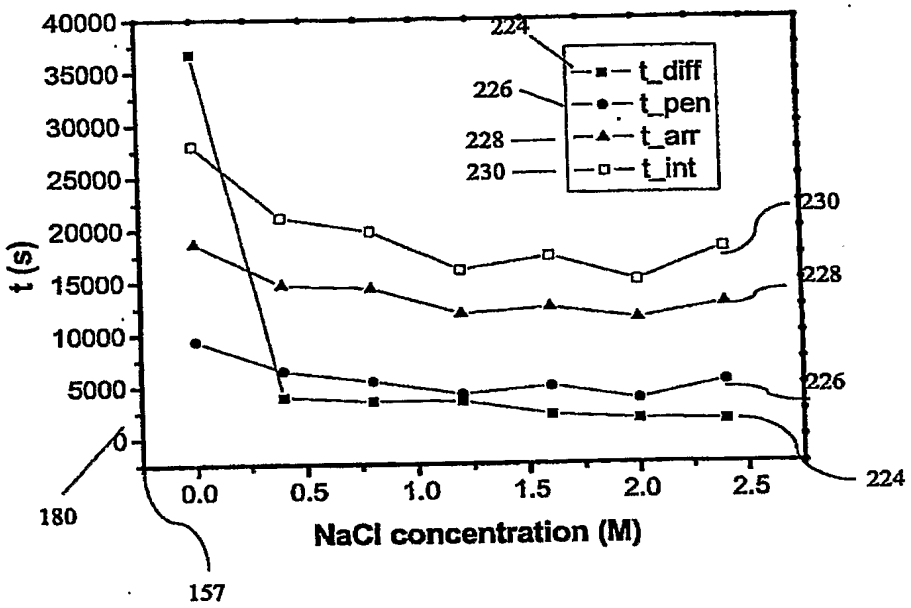

FIG. 11

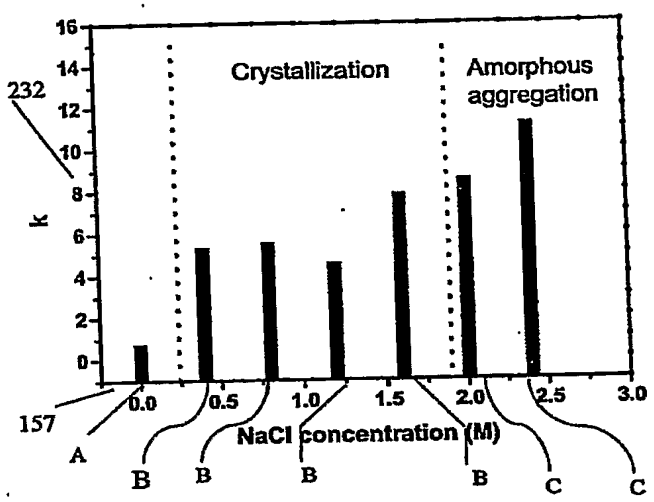

FIG. 12